Challenges for Allergy Diagnosis in Regions with Complex Pollen Exposures

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Abstract Over the past few decades, significant scientific progress has influenced clinical allergy practice. The biological standardization of extracts was followed by the massive identification and characterization of new allergens and their progressive use as diagnostic tools including allergen micro arrays that facilitate the simultaneous testing of more than 100 allergen components. Specific diagnosis is the basis of allergy practice and is always aiming to select the best therapeutic or avoidance intervention. As a consequence, redundant or irrelevant information might be adding unnecessary cost and complexity to daily clinical practice. A rational use of the different diagnostic alternatives would allow a significant improvement in the diagnosis and treatment of allergic patients, especially for those residing in complex pollen exposure areas.

Keywords Allergy diagnosis · Pan-allergen · Component-resolved diagnosis · Pollen allergy · Food allergy

Introduction

In contrast to tropical areas, where mites are the leading and almost sole cause of respiratory allergy, in temperate regions, such as Europe or North America, pollen plays a major role in allergic patient sensitization.

There are important differences in pollen sensitization profiles. Grasses, mainly from the Poaceae family, are the most frequent pollen sensitizers. In north and central Europe, birch pollinosis ranks second in allergy triggering and together with grasses account for most of the pollen-related allergic symptoms [1].

Mediterranean and dry areas present quite different pollen exposure patterns. In addition to grasses, other pollens, such as Oleaceae, Amaranthaceae, Cupressaceae, Asteraceae, and Urticaceae, play a significant clinical role [2, 3]. In North America, there are pollen regions coincident with European ones with the added complexity of greater tree diversity and a high incidence of ragweed pollinosis [4, 5]. Ragweed pollinosis was exported at the beginning of the twentieth century to central Europe and has steadily increased since then [6, 7]. Other pollinosies caused by plants originating in desert regions are also of increasing importance in semi desertic areas.

Pollen extracts used for diagnosis consist of the complex mixtures of major allergens (sensitizing more than 50 % of the patients allergic to a particular pollen), minor allergens, with a lower prevalence, pan-allergens, and non-allergenic molecules [8•]. Major allergens are relatively abundant molecules that are good markers of primary sensitization in general. Minor allergen may be of importance for identifying differential, often more severe, clinical phenotypes and rarely sensitize patients independently from major allergens [9].

Pan-allergens are highly conserved molecules present in practically all pollens (and, in some cases, in vegetable foods) and are highly cross-reactive [10–12]. As a consequence,

patients sensitized to pan-allergens will yield positive extract-based diagnosis to multiple pollens and foods.

Biological standardization consist of assigning a potency to an extract that is related to the biological activity in a dose-response skin test procedure performed on a representative sample of allergic patients [13–16]. While frequent sensitizers, such as grasses or birch, can be easily standardized, pollens with low prevalence in the allergic population are difficult to standardize and can be biased, at least partly, to pan-allergen-sensitized populations [2]. It can be estimated that at least 20 % of pollen-allergic patients are sensitized to pan-allergens and will yield positive skin responses to almost any pollen extract. These extracts will be highly unspecific, and they add further noise to an already-complex diagnostic situation.

The combination of complex pollen exposure to many pollens, pan-allergen prevalence, and poorly standardized extracts often makes the correct diagnosis of allergic patients resident in these areas by extract-based diagnosis almost impossible [17]. Thus, there is a misperception of extreme polysensitization and an urgent need to simplify and improve diagnosis in daily clinical practice.

The increasing availability of new in vitro diagnostics incorporating single-allergenic molecules either as single-allergen components, multiplexed, or in microarrays has impacted clinical practice in allergy diagnosis allowing a more accurate identification of leading sensitizers [18, 19••, 20–25]. However, there are still some problems that must be overcome. The high price of most of the available methods makes it desirable to define a rational use, as many health care providers cannot finance their indiscriminate use. Incomplete, redundant, or inadequate molecular panels, non-automated procedures, non-validated allergens, and poor-quality molecules make mass application of these protocols difficult and reduce their efficacy since an expert scientific background is required to make a reliable diagnosis. We will review available information in order to identify pragmatic diagnosis approaches using a combination of existing procedures and technologies to improve current practices for specific allergy diagnosis with a focus on patients living in complex pollen regions.

Skin Prick Testing Diagnosis

Skin prick testing constitutes the primary allergy diagnostic tool, providing quick and relevant results, being also relatively inexpensive. Introducing single-allergen components in skin prick test, daily clinical practice would constitute a significant improvement. However, regulatory burdens make a massive molecule-based skin prick test (SPT) commercialization almost impossible, at least in the near future. However, it would be feasible to introduce a limited number of new approaches that might allow the identification of pan-allergen reactors [2, 26, 27]. By doing this, the subset of patients that cannot be diagnosed by SPT might be quickly identified, allowing a better diagnosis in the remaining patients. Profilin and lipid-transfer protein (LTP) SPT diagnostics are already available in some European countries. Profilin is the most frequent pan-allergen sensitizer, and it is normally linked to grass pollinosis [3, 28•] with a prevalence increasing along grass pollen gradients, that is, with increased grass pollen counts that includes both pollen peaks and pollinization period and is associated with a characteristic food allergic phenotype that might also be a good indicator of grass pollen allergy severity. In some areas, profilin prevalence reaches 60 % of pollen-allergic patients. In regions with a lower profilin prevalence but with multiple pollen allergies, testing with profilin might allow the identification of 10–15 % of patients that cannot be diagnosed by SPT and would consequently enable a quick diagnosis of most patients by extract-based skin prick test.

If pan-allergen diagnostics are not available, a good alternative would be to use an extract made from a pollen that is irrelevant for the resident population, such as a palm tree pollen extract [27]. Positive response to such an extract would mark unspecific responses to multiple pollens, so alternative diagnostic procedures might be advisable. Negative palm tree pollen response indicates a reliable SPT procedure to the remaining pollens. Unspecific SPT responses might be due to pan-allergens, residual pollen cross-contamination, or even to cross-reactive carbohydrate determinant (CCD) reactivity in some cases.

Apart from controls for unspecific reactions, the selected panel of pollen extracts should be designed according to the pollens in the area avoiding the use of redundant cross-reactive extracts [29].

Major Allergen Markers and Cross-Reactivity

Major allergen sIgE-based diagnosis is an extremely valuable diagnostic tool. First, major allergens sensitize most allergic patients to a particular pollen; second, and more importantly, diagnoses and specific therapies are standardized and adjusted in relation to the major allergens [30–35]. As a consequence in most cases, major allergen-based diagnosis will be enough to correctly diagnose and, thus, adequately select extracts to treat more than 90 % of allergic patients. Patients that are not sensitized to major allergens or pan-allergens do exist but normally represent less than 10 % of the allergic population, and there is little evidence of the effect of major allergen-adjusted therapies in this type of patients.

The following is a brief summary of both major and clinically relevant minor pollen allergens and their cross-reactivity that can be used for the diagnosis of patients resident in complex pollen areas

Grasses

Grass allergy to the Pooideae subfamily can basically be diagnosed with two major allergens Phl p 1 and Phl p 5 [36–40]. All major allergens belonging to this subfamily present a very high similarity degree [41, 42], and there is scientific evidence that a single species or even single major allergen isoform can be successfully used for patient treatment [43–46].

About 10 % of grass-allergic patients can be negative to Phl p 1 and 5 and sensitized to other grass allergens such as Phl p 2, Phl p 4, or Phl p 11.

Prevalence of Phl p 1 is very high (close to 90 %), while Phl p 5 prevalence, depending on the areas, ranks from 50 to 80 %. Pan-allergen profilin behaves as a minor grass allergen, and its prevalence is associated with Phl p 5 [3]. Recently, severe profilin-mediated food allergy reactions have been described [28•]. Interestingly, sIgG4/sIgE levels to Phl 5 and Phl p 2 were lower in patients suffering severe reactions. Profilin sensitization and food allergy symptoms induced by profilin correlates with grass allergy severity [28•]. It is important to mention that profilin sIgE readouts should be made globally without taking into account sIgE values to the different profilins to assign profilin sensitization procedence. In some diagnostic systems, such as ISAC-CAP, several profilins are included with very different performance. For instance, rPhl p 12 normally shows a much lower sensitivity, probably in relation to inadequate isoform election or incorrect folding.

Other allergenic grasses belonging to Panicoideae (Johnson grass and Bahia grass) or Chloridoideae (Bermuda grass) lack group 5 allergens but do have group 1 allergen that displays a high (around 70 %) sequence identity with Phl p 1. In extensive epidemiological studies testing both Cyn d 1 and Phl p 1, very few patients positive to Cyn d 1 and negative to Phl p 1 were found [47]. To date, there is no clinical evidence for the incremental effect of adding any of the non-Pooideae subfamily species in immunotherapy formulas in grass-allergic patients. Biological standardization procedures performed on these latter species will overdose group 1 allergens compared to Pooideae, where group 5 allergen greatly contributes to biological potency. Basically, for species lacking group 5, the extracts will be tested in the same grass-allergic population, but the concentration of group 1 allergens will be increased to compensate for group 5 absence as the target of biological standardization is to induce a defined quantitative biological response (wheal area). Consequently, up to 40 % of grass-allergic patients that are group 1 monosensitized might react stronger to Bermuda, Bahia, or Johnson grass extracts, without any clinically relevant implication. In commercial platforms, rPhl p 1 is included together with nCyn d 1; thus, strong CCD reactors might yield positive reactivity to nCyn d 1 and negative to rPhl p 1, the former being unspecific. Moreover, we will be comparing a single isoform with the multiple isoforms included in a natural allergen preparation. To date, there is no major allergen marker commercially available for Johnson and Bahia grass (Sor h 1 and Pas n 1).

Polcalcins, a group of pan-allergens that includes Phl p 7, sensitize between 5 and 10 % of pollen-allergic patients and are not associated with any particular pollen. It is a diagnostic confounding factor and, when found as a sensitizer together with profilin, is linked to more complex sensitization profiles and many years of disease evolution [2]. As a consequence, it should be incorporated into diagnostic panels for CRD.

Birch/Oak Pollen Allergy

Bet v 1 is the best marker of PR10 related tree pollen allergy. In the US, where there is an important contribution of oak tree pollen to tree pollen-allergic phenotypes, it might be interesting to test the homologous allergen Que a 1 as well, which is not currently commercially available in component-resolved diagnostic (CRD) platforms.

Several PR10 molecules are incorporated in some arrays used for CRD, both pollen and food related. Therefore, a global interpretation should be performed evaluating food allergy in the context of PR10 allergy. Practically all patients with strong Bet v 1 recognition will yield a positive response to most PR10 allergens due to cross-reactivity to Bet v 1 (or Que a 1)

Oleaceae

Major olive pollen allergen Ole e 1 can be used as a marker of sensitization. Ole e 1 is fully cross-reactive to ash tree major allergen Fra e 1, so olive extract can be used to diagnose and treat ash tree pollen-related allergy, which is relatively prevalent in some areas of central Europe [48, 49]. The same similarity degree is also shown by the other six sequences recently analyzed in ash pollen corresponding to the counterpart in olive pollen [50]. The broad family of Ole e 1-like proteins contains members in many if not all types of pollens, but neither their relations nor their allergenic relevance are as close as those between Ole e 1 and Fra e 1.

Patients living in areas overexposed to olive tree pollen become sensitized to minor allergens, displaying a different and more severe phenotype. The inclusion of minor allergens such as Ole e 7 (belonging to non-specific lipid-transfer protein (nsLTP) family) and Ole e 9, olive pollen β-glucanase, is necessary to identify these patients [51]. In fact, in these extremely exposed areas, there are a significant proportion of patients negative to Ole e 1 and positive to Ole e 7. These could even present negative skin prick test responses to olive pollen because of the low concentration of this minor allergen in the whole pollen extracts [52].

Cupressaceae

Cup a 1, Cup s 1, and Jun a 1 are major allergens of different cypress species with a very extensive cross-reactivity [53]. As a consequence, any of them can be used to identify cypress sensitization. In Japan, where Japanese cedar allergy is very prevalent [54], Cry j 1 should also be added. To date, there is no efficient procedure to clone and express cypress allergens, and as a consequence, natural forms are incorporated in CRD procedures with the corresponding lack of specificity due to CCD cross-reactivity [55, 56]. In the interpretation of these results, other glycosylated molecules such as nJug r 2, nPhl p 4, or the MUXF3 CCD marker should be taken into account.

Amaranthaceae (*Salsola* and *Chenopodium*)

The relevance of these allergies, typical from desert regions, is increasing in countries as Spain because of their resistance to saline soils and dryness. Sal k 1 is a marker of *Salsola* allergy (Russian thistle) [57] that is the leading sensitizer of this pollen family. The inclusion of an allergen such as Che a 1 [58], cross-reactive between the different *Salsola* and *Chenopodium* species, contributes to the diagnosis of Amaranthaceae allergic patients.

Asteraceae (*Artemisia*, *Ambrosia*)

Asteraceae pollen, particularly ragweed, is a main cause of clinical allergy over extensive North American regions. Art v 1 for *Artemisia* species and Amb a 1 for *Ambrosia* (ragweed) allow a correct identification of most of Asteraceae-sensitized patients [59, 60]. Art v 3 is a lipid-transfer protein of *Artemisia* pollen partly cross-reactive with Pru p 3, the main LTP syndrome marker. Sensitization to Art v 3 in the absence of Art v 1 should be interpreted in the context of LTP allergy.

Urticaceae (*Parietaria*)

Par j 2 is the main marker of *Parietaria* sensitization [61], highly prevalent in Mediterranean countries. In spite of being an nsLTP, as also occurs with the olive pollen nsLTP, Ole e 7, Par j 2 is not cross-reactive to any other known allergen [62].

Plantaginaceae (*Plantago*)

Plantain allergy is difficult to assess as the pollen season overlaps with that of grasses. There is a specific marker, Pla l 1 [63], an Ole e 1-like member, that is available for specific diagnosis. Epidemiological studies suggest that plantain allergy is relevant in some areas [2, 3].

Platanaceae (*Platanus*)

Pla a 1 and Pla a 2 [64, 65] identify *Platanus* sensitization. In areas with very high exposure to this pollen, as Barcelona [66], Pla a 3, the LTP from the pollen [67], sensitizes some patients. As this protein has partial cross-reactivity with food LTPs, its sensitization should be evaluated in the context of LTP allergy as to Art v 3.

Non-specific Lipid-Transfer Proteins (nsLTPs)

LTP-mediated allergy is a complex food-pollen syndrome with a particularly high incidence in Mediterranean countries. Given the currently active research on LTPs, a better understanding of the real worldwide incidence of LTP allergy will be available in the coming years. As an example, *Artemisia*-related LTP sensitization has recently been described in northern China [68], suggesting that LTP allergy is relevant in other regions apart from the Mediterranean border. Peach LTP, Pru p 3, [69] is, in most cases, the leading LTP allergen. While some patients are only reactive to a limited number of LTPs (mainly from Rosaceae), some others develop sensitization and side reactions to multiple LTPs, in what is known as the LTP syndrome [70, 71]. Exposure to cross-reactive pollen containing LTPs seems to correlate with a higher recognition LTP pattern. Currently, a panel of different LTPs from foods and pollens is available for diagnosis, but the interpretation of these results is often complex.

Sensitization and Clinical Relevance

SPT-based epidemiological surveys together with aerobiological pollen data [1, 7, 68, 72–75] in correlation with allergic symptoms have been the basis for defining clinically relevant sensitizations. Apart from the previously described problems associated with extract-based diagnosis, it is not always possible to define the relevant allergens for a particular population and even more difficult to make a choice of the clinically relevant sensitizers for a particular patient. Coincident pollen seasons, long and discontinuous pollinization, pollens with low aerovagant capacity but able to sensitize by close contact or in areas with sustained strong winds and different sensitization threshold for different populations can at least partly explain the complexity of the problem.

CRD offers a new tool to understand the complex dynamics underlying patient sensitization. In a systemic epidemiological study performed throughout Spanish territory, more than 2000 patients homogenously distributed over the territory were sampled from the allergic population [2, 3]. The results obtained allowed the main sensitizers to be identified in the

Table 1 First three sensitization frequencies (%), accumulated patients covered (%), and profilin prevalence (%) in Spain. Results are given by provinces, administrative territories of an average surface of 10,000 km^2

		G	O	P	S	A	Pla	B	G + O	G + Pl	O + S	G + C	O + P	G + S	G + B	G + A	O + C	P + C	G + P	G + O + 1	G + O + 2	LTP (Pru p 3)	Total	Profilin
Andalucia	Almeria	11	28								33												72	5
	Cadiz	10	39						38														87	8
	Cordoba	15	38						36														89	20
	Granada		57						11				15										88	9
	Huelva	24							32											14			70	19
	Jaen	18	55						18														91	11
	Malaga		58								10		11										79	15
	Seville	20	32						15														67	14
Aragon	Huesca								12												19	12	43	29
	Teruel																			8	16	8	32	24
	Zaragoza	43			6				12														61	10
Asturias	Asturias	59							10	20													89	23
Baleares	Baleares	16	16	13																			45	8
Canarias	G. Canaria	16				40														8			64	4
	Tenerife			40		20												8					68	
Cantabria	Cantabria	65								12		9								19			86	26
Cast. Leon	Avila	23							26														68	20
	Burgos	63							8	8													79	18
	Leon	60								20						12							92	32
	Palencia	65	4																		7		76	28
	Salamanca	42							20											6			68	12
	Segovia	40							23			7											70	20
	Soria	55							5			12											72	10
	Valladolid	53							9	11													73	14
	Zamora	51							18	13													82	22
Cas. la Mancha	Albacete		27								10												57	15
	Ciudad real		39						24		10												73	2
	Cuenca	14							12											18			44	16
	Guadalajara	30			6				26														62	22
	Toledo	16	25						16														57	22
Cataluña	Barcelona	23		11			12																46	6
	Gerona										16									32			48	24
	Lerida	20			23									17									60	6
	Tarragona		33						10								10						53	
Extremadura	Badajoz	52	6						27														85	36
	Caceres	45	9						11														65	39

Table 1 (continued)

		G	O	P	S	A	Pla	B	G+O	G+Pl	O+S	G+C	O+P	G+S	G+B	G+A	O+C	P+C	G+P	G+O+1	G+O+2	LTP (Pru p 3)	Total	Profilin
Galicia	la Coruña	62	12					12		12													86	17
	Lugo	51		22						22													95	24
	Orense	61								15					13								89	28
	Pontevedra	67		7						9													83	32
La Rioja	La Rioja	53							9										13				75	17
Madrid	Madrid	33							21										15				69	15
Murcia	Murcia	16									22								8				46	5
Navarra	Navarra	51							13			4											68	13
Pais vasco	Alava	63							11	16													90	26
	Gipuzkoa	68	14						14														96	9
	Vizcaya	76								15								5					96	25
C. Valenciana	Alicante		12		14						22												48	6
	Castellon	18	30	17																			65	11
	Valencia		40	12					21														73	10

G grass, *O* olive, *C* cypress, *P Parietaria*, *S Salsola*, *A Artemisia*, *Pl Plantago*, *Pla Platanus*, *B* birch, *G + O + 1, 2* combinations of three or four sensitizations including grass and olive

different regions, according to prevalence to major and minor allergens.

An alternative way of analyzing the data consists in classifying resident patients according to their sensitization profiles. By clustering patients in such a way and considering only the first three sensitization frequencies, we constructed Table 1. This table also shows the prevalence to profilin and the coverage of the first three frequencies by region.

When analyzing the data, three clinically relevant conclusions can be highlighted. First, as can be seen in Fig. 1, three sensitization clusters (sensitized to grass or olive alone or the double sensitization to grass and olive) cover most of the patients. In fact, in many areas, these frequencies cover more than 90 % of patients with seasonal allergy. This was not always evident as high exposure levels either to grass or olive pollen cause minor allergen and pan-allergen sensitization, exemplified in the table by profilin prevalence data. From a clinical perspective in many areas, the diagnostic problem of pollen-allergic patients is limited to choosing between grass, olive, or both [76]. There are specific tools such as Phl 1, Phl p 5, Ole e 1, and Ole e 7, which together with pan-allergen molecules will facilitate the diagnosis at an affordable price.

Interestingly, in these studies, the other pollens, such as Cypress or *Salsola*, in spite of sensitizing more than 50 % of the patients, hardly ever monosensitize patients. The progression of disease in sensitization in a particular region can also be analyzed. As an example in Madrid, the first three frequencies in this order are grass alone, grass/olive, and grass/olive/cypress. This finding suggests that grass pollen is the most clinically relevant pollen in the area. The natural evolution of sensitization in a particular region might help to choose clinically dominant allergies or may help in the design of clinical trials. For species that almost never monosensitize the population, clinical trials might be designed to check the incremental benefit of adding an allergen to, for example, a grass-based therapy.

In areas with low grass pollen counts, patients are still sensitized to grasses but with a more complex profile. In these regions, located along the Mediterranean border and dry inner areas, patients are truly polysensitized. The first three frequencies only covers a fraction of the allergic patients. Based on our experience, we suggest that a cut-off value of 70 % (patients covered by the first three sensitization frequencies) could define the border between simple and complex pollen areas. In complex areas, as grass pollen counts are relatively low, profilin prevalence is also low, and thus, extract-based SPT offers more value than in areas with a much lower complexity, but higher pan-allergen prevalence. In areas with close to 90 % of patients monosensitized to grasses, but with a prevalence of pan-allergens in the range of 30 %, extract-based diagnosis is only causing diagnostic failure in 30 % of patients.

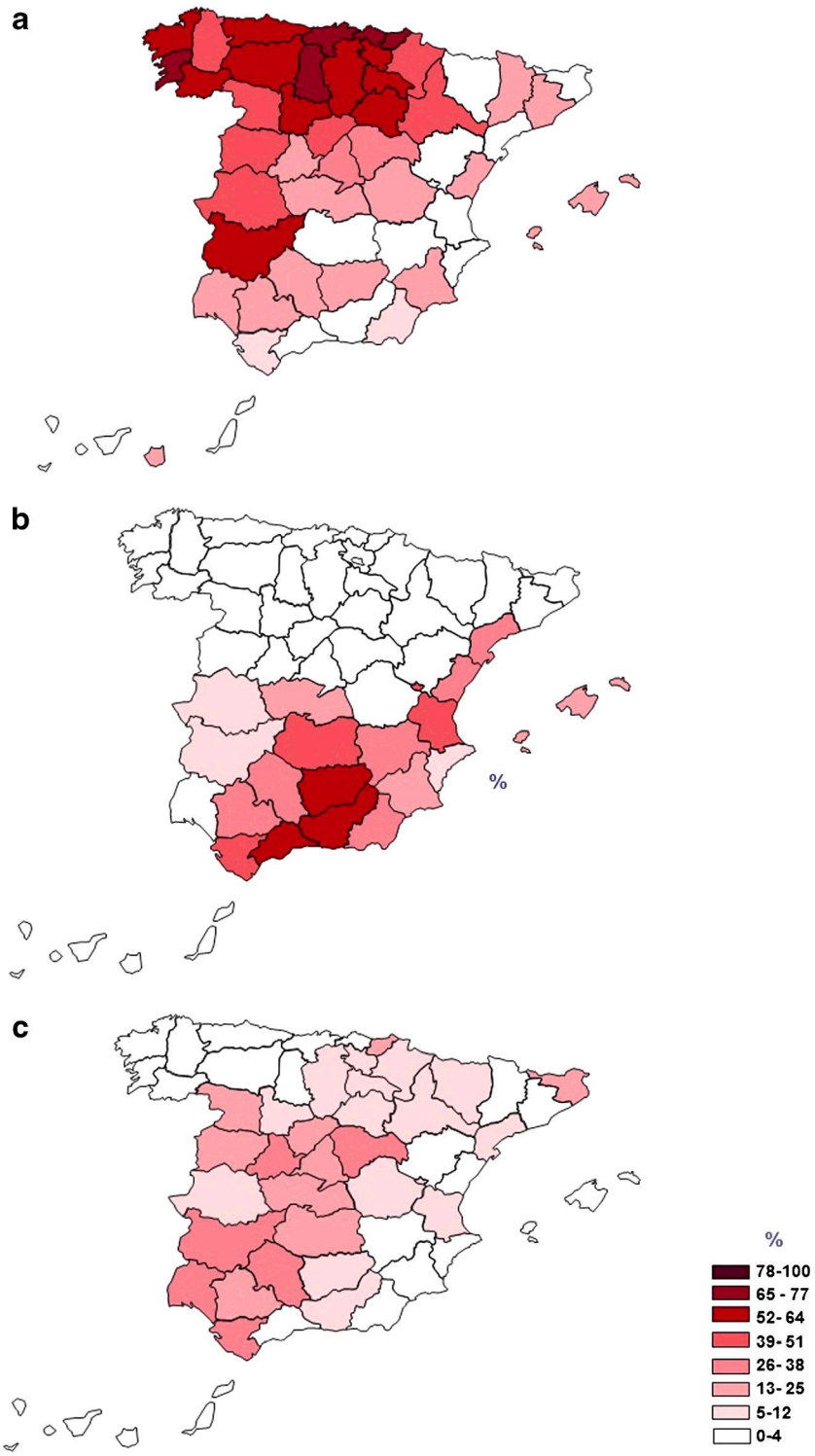

Fig. 1 Geographical distribution of seasonal allergic patients that are **a** grass monosensitized, **b** olive monosensitized, and **c** co-sensitized to grass and olive. Percentages of patient color codes are shown on the right

In dry areas with extensive orchard tree cultivation (Teruel, Huesca) especially peach trees, a significant fraction of patients with seasonal respiratory symptoms are monosensitized to Pru p 3, the major allergen of peach and a severe food allergen. This finding demonstrates a new entry gate to fruit LTP-mediated allergy and supports the link between food and respiratory allergies. In fact, the three most common allergies affecting adults are related to PR10, LTPs, and profilin, all of them connected to respiratory allergies

Knowing in advance the dynamics of sensitization will help to define local diagnosis algorithms which will allow an improved and effective diagnosis of allergic patients [2].

CRD is an increasingly appreciated tool that should complement traditional diagnostic procedures to improve daily clinical practice. Besides available methods, all of them complex, time consuming, and relatively expensive, there is a need for quick, inexpensive in vitro diagnostic tests adapted to the different patient sensitization profiles. For example, a test for Phl p 1,5, Ole 1, Ole e 7, profilin, and polcalcin would offer a high diagnostic resolution capacity in most of the Spanish territory. In a similar way, Phl p 1, 5 , Bet v 1, profilin, and polcalcin would simplify pollen diagnosis in north and central Europe. This last panel with the inclusion of Amb a 1 would significantly improve pollen allergy diagnosis in North America. In this context, recent product diagnosis development such as multiplexed assay recently commercialized [77] might be of help.

Conclusions

Available data support that patients are usually sensitized to not more than three or four primary sensitizers and that, normally, it is possible to select one or two clinically relevant ones for specific treatment. Correct diagnostic approaches to identify clinically relevant sensitizers are possible with the existing tools available today.

Sensitization analysis alone will never be enough as it is necessary to carry this out in a clinically relevant context. For this purpose, aerobiological data interpretation, knowledge of patient sensitization patterns, and a deep understanding of the connection of allergens with associated allergic diseases is mandatory and constitutes the basis of allergy speciality.

Compliance with Ethics Guidelines

Conflict of Interest Domingo Barber, Araceli Díaz-Perales, Mayte Villalba, and Tomas Chivato declare that they have nothing to disclose.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance

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